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Species determination of ulvoid algae through genotyping; what are the environmental implications?

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SPECIES DETERMINATION OF ULVOID ALGAE THROUGH GENOTYPING: WHAT ARE THE ENVIRONMENTAL IMPLICATIONS?

by

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ABSTRACT

Ulva is a genus of marine green algae native to many of the world's coastlines and is especially difficult to identify via traditional methods such as dichotomous keying. This project aims to streamline taxonomic classification of *Ulva* species through DNA sequence analysis. Local samples of *Ulva* were obtained from Puget Sound, Seattle, WA, and two target genes (*rbcL* and *its1*) were amplified via PCR and sequenced for comparative analysis between samples. Ulvoids have a detrimental impact on marine ecosystems in the Pacific Northwest due to their role in eutrophication-caused algal blooms, and reliable identification can help inform conservation efforts to mitigate these effects. An appendix discusses this research in the context of the Christian faith.

INTRODUCTION

The identification of plant species has long been practiced using keys or atlases and is typically based on appearance alone. A more precise identification can be made using DNA sequencing, which could be crucial in finding medicinal plants, mapping the locations of specific species, or distinguishing between two visually identical taxa (i.e., cryptic species). Ulvoid algae, a genus of marine green macroalgae (Fig. 1), is notoriously difficult to identify by dichotomous key; the species-level taxonomic classification is difficult to achieve based on morphological appearance. DNA sequence analysis has been used with ulvoid algae before and has proven useful in revealing the true classification of cryptic species (Hayden, 2003; O'Kelly, 2010). In this study, taxonomic identification was attempted through DNA extraction, PCR amplification, and sequencing of key genes known to exist in our target species. The genes of interest in this study were the internal transcribed spacer gene (*its1*), which is spacer DNA found in the eukaryotic ribosome, and the large-chain gene coding for ribulose-1,5-biphosphate carboxylase/oxygenase or RUBISCO (*rbcL*), a crucial enzyme in photosynthesis/carbon fixation which is found in chloroplasts. Different species of *Ulva* contain genetically distinct DNA sequences, and these differences should allow for highly accurate identification based on comparative analysis with published sequences.

Figure 1. Four scanned herbarium presses of *Ulva* collected for analysis in this study. Specimens were pressed onto herbarium paper for preservation after pieces were taken for DNA extraction.

METHODS

Sample collection and preparation

Thirteen samples of green algae from the genus *Ulva* were collected from Me Kwah Mooks park on Puget Sound, Seattle, Washington (47.563450, -122.406436), and bagged using methods from O'Kelly et al. (2010). At Seattle Pacific University, the samples were cleaned with a toothbrush under sterile water to remove epiphytes. A small piece of each sample (<200 mg) was crushed in mortar and pestle and transferred into microcentrifuge tubes for lysis.

Genomic DNA extraction

A Qiagen DNEasy Plant Mini Kit was used to extract genomic DNA from each sample, per the kit instructions (Qiagen, Hilden, Germany). DNA concentration in each extraction was quantified on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Gene amplification

PCR was used to amplify the target genes. Invitrogen 2x PCR High Fidelity Supermix with PCR enhancer (Invitrogen, Waltham, Massachusetts, USA) was used in combination with primers listed in Table 1, at 0.5 µM each. One µL of genomic template DNA was used in each reaction. *rbcL* PCR reactions included two additional internal primers, developed by Guidone et al. (2013) for improved target accuracy. PCR cycling consisted of an initial denature at 95°C for 2 minutes, followed by 35 cycles of 95°C for 10 seconds, 56.75°C for 10 seconds, and 68°C for 45 seconds, with a final extension at 68°C for 5 minutes for *rbcL*. *its1* cycling included 3 min at 94°C, 35 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 90 sec, with a final extension of 7 min at 72°C.

Gel electrophoresis

PCR products were visualized using a ProteinSimple imager and FluorChem Q software (ProteinSimple Incorporated, San Jose, California, USA) in 2.0% agarose gel run for one hour at 100 volts, stained with 1x Sybr Safe dye (Invitrogen, Waltham, Massachusetts, USA).

Table 1. Primer sequences for PCR amplification of targeted genes. Adapted from [a]Manhart (1994), [b]Guidone et al. (2013), and [c]Hayden et al. (2003).

Primer	Nucleotide sequence	Target gene
RH1[a]	5' ATGTCACCACAAACAGAAACTAAAGC 3'	*rbcL*
1385r[a]	5' AATTCAAATTTAATTTCTTTCC 3'	*rbcL*
rbc349F[b]	5' TTATTTACTTCAATTGTAGGGAACG 3'	*rbcL*
rbc1027R[b]	5' CGCATTAARTCAACGAAACCTAAAGT 3'	*rbcL*
18S1505[c]	5' TCTTTGAAACCGTATCGTGA 3'	*its1*
ENT26S[c]	5' GCTTATTGATATGCTTAAGTTCAGCGGGT 3'	*its1*

RESULTS

The average DNA concentration in the 13 extractions was 21.17±5.40 (mean±SE) ng/uL. Their average 260/280 absorbance ratio was 1.61±0.07 (mean±SE) and the 260/230 absorbance ratio was 1.01±0.21 mean±SE). No bands representing PCR product were visualized with gel electrophoresis except two possible traces of a product (Fig. 2).

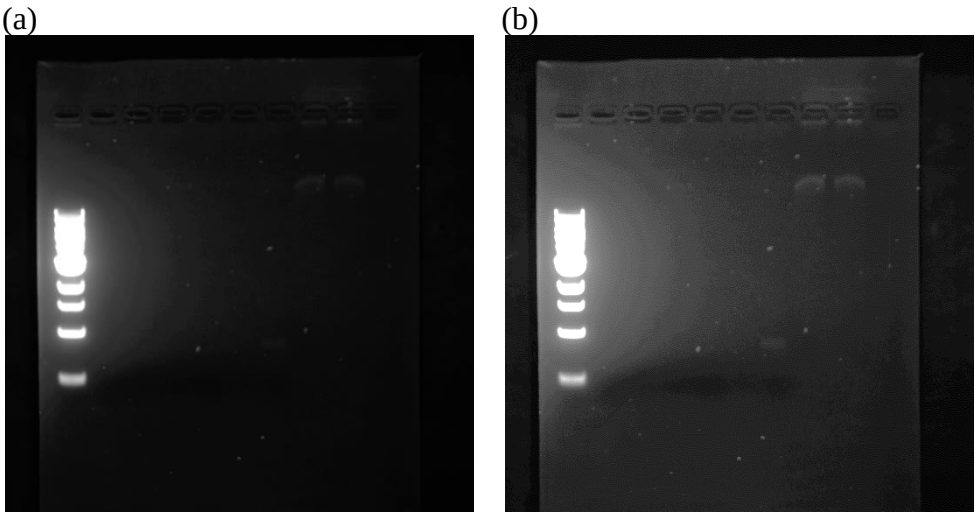

Figure 2. Agarose gel with 1kb DNA ladder on the left (a). Overexposed gel image with 1kb DNA ladder on the left, and two faint bands (*rbcL*) on the right side of the gel (b).

DISCUSSION

Although PCR did not clearly amplify the targeted regions (Fig. 2) the nanodrop readings confirm that DNA was successfully extracted from the samples of algae. The DNA concentration in the samples matches the specification of the Qiagen extraction kit, and the 260/280 absorbance reading indicate little protein contamination in the extract (~1.80 is optimal). One issue brought about by the nanodrop readings is the low 260/230nm absorbance ratio (~2 is optimal), which can indicate contamination by salts, EDTA, or guanidine hydrochloride. This contamination could have resulted from inadequate cleaning of the samples before DNA extraction, where seawater containing salts and other contaminants was still present in the samples when they were lysed.

Table 2. A subset of PCR protocols attempted for *rbcL* and *its1* gene amplifications.

Gene	**Components**		**Cycle**	
	Components	**Volume (µL)**	**Temperature (°C)**	**Time (min)**
rbcL	SuperMix	15	94	3
	10µM forward primer	1	94	1
	10µM reverse primer	1	45	3
	Template DNA	3	65	3
			repeat 2-4 35x	
			60	7
			4	infinity
rbcL	SuperMix	15	94	3
	10µM forward primer	1	94	1
	10µM reverse primer	1	42	2
	10µM forward enhancer primer	1	65	3
	10µM reverse enhancer primer	1	repeat 2-4 35x	
	Template DNA	1	65	7
			4	infinity
rbcL	SuperMix	17	94	3
	10µM forward primer	1	94	1
	10µM reverse primer	1	45	3
	Template DNA	1	65	3
			repeat 2-4 35x	
			60	7
			4	infinity

rbcL	SuperMix	15	95	2
	10µM forward primer	1	95	0.17
	10µM reverse primer	1	56.75	0.17
	10µM forward enhancer primer	1	68	0.75
	10µM reverse enhancer primer	1	repeat2-4 35x	
	Template DNA	1	68	5
			4	infinity
its1	SuperMix	17	94	3
	10µM forward primer	1	64	0.5
	10µM reverse primer	1	51	0.5
	Template DNA	1	72	1.5
			repeat 2-4 35x	
			72	7
			4	infinity
its1	SuperMix	15	94	3
	10µM forward primer	1	94	0.5
	10µM reverse primer	1	56	0.5
	Template DNA	3	72	1.5
			repeat 2-4 35x	
			72	5
			4	infinity

Regardless of this potential contamination, I suspect the main issues occurred in the PCR cycling. Many iterations of differing temperatures, durations, and number of cycles from various papers, reagent specifications, and calculated primer annealing temperatures were attempted, but no bands were seen in gel electrophoresis (Table 2). Guidone et al. (2013) proposed additional *rbcL* primers which were also added to the PCR master mix for this gene, but as of yet, the amplification has been unsuccessful. Next steps include reoptimization of this cycling after seeking input from specialists in the field of genetics, including Dr. Brian Wysor, a specialist in molecular identification of plant species.

Making these changes to pursue streamlined DNA identification of ulvoid algae may inform conservation efforts involving algal blooms. *Ulva* can form algal blooms in Pacific Northwest (PNW) waters in response to extreme nutrient influx, or eutrophication, which can result in hypoxic conditions and fish kills (Nelson et al., 2008; Nelson et al., 2003). PNW waters are especially at risk for eutrophication and subsequent algal blooms because of the outdated wastewater infrastructure of Seattle. Lake Washington originally served as a sewage receptacle for early Seattle residents, up until the population exceeded the ability for the lake to harmlessly disperse human waste. Increased sewage effluent into surrounding waters caused artificial eutrophication of Lake Washington, which decreased the economic benefit of these waters (Edmonson et al., 1965; Carpenter et al., 1999). More recently, Moore et al (2003) surveyed 30 waterbodies in the Seattle region, and found that many had indicators of eutrophication as a result of sewage effluent. Sewage produced by Seattle residents is not pumped into Puget Sound following secondary treatment, however, tertiary treatment (nutrient removal) is not yet universal, and thus could still contribute to blooms in the marine ecosystem of Puget Sound. Carpenter (1999) discussed how the response to eutrophication is important in restoring maximum economic benefit of the waterbody, so response to eutrophic events and conservation of normal conditions is critical in ecosystems where nutrient disruption can lead to these types of events.

Ulvoid blooms not only result in hypoxic dead zones and fish kills, but species in this taxon can reduce the abundance of other marine plants; *Ulvaria* and *Ulva* may be outcompeting other marine plants for resources such as light, HCO_3^- or O_2 (Nelson and Lee, 2001).

Furthermore, *Ulva*-specific “green tides” (algal blooms) have been linked not only to local eutrophic events but also to the production of toxic compounds (Nelson et al., 2003; Van

Alstyne et al., 2015). These toxic compounds include dimethylsulfoniopropionate (DMSP) cleavage products, dopamine quinones, and ichthyotoxic lipids, which have been shown to kill oyster larvae in PNW marine waters (Nelson and Gregg, 2013).

If nearby marine organisms are not killed by the hypoxic dead zones, sharp increase in toxic compound concentration, or increased resource competition, the pungent smell given off by ulvoid decomposition will (Lovelock, 1982; Frankenstein, 2000).

Streamlined taxonomic identification of bloom-forming ulvoids can inform conservation of local and worldwide waters and mitigation of algal blooms. This molecular technique is more time-efficient and precise compared to dichotomous keying, and applicable to members of the target taxon worldwide. In-the-field sequencing technology and genetic barcoding will ease the identification process further. Streamlined identification through gene sequencing and comparative analysis will be uncomplicated and integral in ecosystem degradation response and conservation.

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APPENDIX I

After all this discussion about the danger of eutrophication to our local and global ecosystems, I am including this appendix to discuss the relationship between the health of the environment and the Christian faith. The study of ecology is defined as the scientific study of the interactions that determine the distribution and abundance of organisms. This word *interaction* brings the study of ecology into the context of every other discipline, scientific and otherwise. The interaction between human activity and algal blooms, for instance, has increased in importance in today's age of dense populations with outdated sewage systems. The interaction between fossil fuel usage and the changing climate has been quantitatively significantly correlated (IPCC AR5, 2014). Over 800 international scientists working in the IPCC (Intergovernmental Panel on Climate Change) agreed that climate change (and the myriad processes that the term covers, including temperature change, extreme weather tendencies, melting glaciers, and rising and acidifying oceans) is very likely to be caused by human activity. This data supports the work of Lynn White, Jr., whose 1967 article in *Science* attributes the ecological crisis our world is experiencing today to the predominant religion of occidental society: Christianity. White proposes that, in scripture, God gives Man the earth to rule over, and from here, man invents science and technology to "exploit the land as he chooses" (1967). This exploitation ruins the earth (with hydrogen bombs, fossil fuels, and sewage and garbage deposition). White tracks this logic through the history of human beliefs through agricultural innovation spanning thousands of years, to the medieval revolution which triggered modern science, to the rise of a strict dichotomy between man and nature. In White's interpretation of Christianity, man has a monopoly over spirit, and therefore exploits the rest of Creation for man's own gain.

Lynn White Jr. is not incorrect; the way occidental society interpreted scripture in the context of creation *did* lead to the anthropogenic degradation of the ecological world. The guilty party is not, however, scripture; humans are at fault. Historical biblical hermeneutics are to blame for the mindset that man has a monopoly over spirit, as White suggests. In his book *For the Beauty of the Earth: A Christian Vison for Creation Care* (2010), Steven Bouma-Prediger rebuts Lynn White's paper through reinterpretation of scripture about divine instructions for the intended interaction between humans and the rest of creation. He reminds his readers that God's covenant is made with all of creation, biotic and abiotic (Genesis 6-9), that God is at the center of all things, rather than humanity (Job 38:1 – 42:6), and that in God's future, heaven and earth are rejoined (Revelation 21:1 – 22) (Bouman-Prediger, 2010). This last passage proposes that our planet is not here for man to exploit and use up until we leave for heaven; in the end, heaven will be here in this same place.

Christians, and all people, were called to action as stewards of creation; to obey Genesis 2:15 by protecting and serving (*abad* and *shamar*) divine creation. The human race is not an innocent population huddling together as our home careens toward irreversible environmental chaos. We are the guilty party, and if we want to continue to inhabit the paradise bestowed to our care, we had better study the *ology* of our *eco*, the science of our home. Algae and eutrophication comprise one miniscule part of this *ology*, a part of the whole that needs to be explored to ensure our future as part of Creation.